

Sickle Cell Trait (HbAS) is Associated with Increased Expression of Erythrocyte Complement Regulatory Proteins CR1 and CD55 in Children

Walter Otieno², Benson B A Estambale^{1§1}, Michael M Odera², Stacey M O Gondi², Joash R Aluoch³, José A Stoute,⁴

¹University of Nairobi Institute of Tropical & Infectious Diseases, Kenya

² US Army Medical Research Unit, Kenya Medical Research Institute, Kisumu, Kenya

³ Maseno University School of Medicine, Kisumu, Kenya

⁴ Department of Medicine, Penn State University College of Medicine
Pennsylvania, USA

Email addresses: WO: wotieno@wrp-ksm.org
BBAE: bestambale@uonbi.ac.ke
MMO: modera@wrp-ksm.org
SMOG: sgondi@wrp-ksm.org
JRA: joashaluoch@hotmail.com
JAS: jstoute@psu.edu

Key words: *Plasmodium falciparum malaria, CR1, CD55, sickle cell trait*

¹ §Corresponding author

Prof. Benson Estambale, University of Nairobi Institute of Tropical and Infectious Diseases,
P.O. Box 19676 – 00202, Nairobi, Kenya
Phone: +254 722 700 185; Email: bestambale@uonbi.ac.ke

Footnotes

There is no conflict of interest for any of the authors of the manuscript due to commercial or other affiliations.

Financial support: work was supported by a training grant from the Fogarty International Center (1 D43 TW06239).

The study was approved by the ethical and scientific review committees at the Kenya Medical Research Institute and the institutional review board at the Walter Reed Army Institute of Research.

A portion of this work was presented at the 54th Annual Society of Tropical Medicine and Hygiene conference (Abstract # 792, ASTMH, 2005).

ABSTRACT

Aims: Erythrocyte complement regulatory proteins, complement receptor 1 (CR1) and decay accelerating factor (CD55) protect red blood cells (RBCs) from complement mediated damage by controlling complement activation cascade and potentially protect RBCs from complement mediated damage that may occur when immune complexes are formed following malaria infection. Given the important role of RBCs in regulation of complement activation, we considered the competence of sickle cell trait RBCs in these functions.

Methods: Children (age 0-192 months; n=116) were enrolled in a nested case controlled study conducted in Kombewa Division, Kisumu west District between October and December 2004. Based on hemoglobin (Hb) type, children were stratified into those with HbAS (n=47) and HbAA (n=69). The 47 HbAS individuals were matched to the 69 HbAA individuals of similar age (± 2 months or ± 24 months for those below or more than 192 months, respectively) at a ratio of 1:1 or 1:2. Circulating CR1 levels and CD levels were quantified using a FACScan cytometer under normal and reduced oxygen saturation.

Results: The mean CR1 copy numbers per RBC was comparable in the two groups. However, between the ages of 49-192 months, the mean CR1 copy numbers per erythrocyte was significantly higher in children who had HbAS compared to those with HbAA ($P = 0.0332$). The mean CD55 levels were comparable between the two groups but after deoxygenation, the mean CD levels in RBCs of individuals with HbAS was significantly higher than in the HbAA ($P=0.011$).

Conclusion: The mean CR1 and CD55 copy numbers per RBC were comparable between the two groups under normal and reduced oxygen saturation. Beyond the age of 49 months, the CR1 copy numbers was higher in the HbAS compared to HbAA and this was also true for CD55 levels under deoxygenated conditions. Taken together, these results demonstrate that in the younger age groups, the protection afforded by HbAS against severe manifestations of malaria may be due to other factors other than complement regulatory proteins but beyond the age of 49 months, this protection may be partly due to the high CR1 copy numbers in the HbAS individuals.

1. BACKGROUND

The erythrocyte complement regulatory proteins, Complement Receptor-1 (CR1), Decay Accelerating Factor (DAF/CD55) and Membrane Inhibitor of Reactive Lysis (MIRL/CD59) are important in protecting RBCs from complement mediated damage and in controlling the complement activation cascade. Erythrocyte CR1 and CD55 are important determinants of

77 malaria susceptibility [1-3]. The interaction of *P.falciparum* Erythrocyte Membrane Protein-1
78 (PfEMP-1) expressed on *P. falciparum* infected cells with CR1 on uninfected erythrocyte results
79 in rosetting leading to obstruction of blood flow and hence cerebral malaria [1]. Rosetting has
80 been shown to be more common in isolates from children with complicated malaria and its
81 frequency is lower in isolates from blood group O patients than from blood group A or B [4, 5].
82 RBC CR1 deficiency is common in malaria –endemic region of Papua New Guinea and
83 heterozygotes for the CR1 low-expressions allele (HL) are significantly protected from severe
84 malaria possibly through reduced rosetting [6]. The selection of rosette reducing polymorphisms
85 in populations with high malaria mortality is strong evidence that CR1-mediated rosetting plays
86 a causal role in the pathogenesis of severe malaria [6].

87 Studies carried out in Western Kenya where malaria is holoendemic and malarial anaemia is the
88 main manifestation of severe malaria in children have suggested that low CR1 and CD55 levels
89 on red cells of severe malarial anaemia cases increase their susceptibility to phagocytosis and
90 complement mediated lysis [2, 3]. The CR1 and CD55 levels were shown to be corrected with
91 treatment and transfusion and remained high long after the maximum expected lifespan of the
92 donor RBCs suggesting that these changes were acquired [2, 3]. In Thai patients with severe
93 malaria, a significant higher frequency of the Low expression genotype [LL] was observed. The
94 LL genotype is associated with decreased CR1 copy numbers per erythrocyte and this may
95 result in decreased clearance rate of immune complexes from the circulation which then
96 predisposes one to malaria through increased deposition of immune complexes on red cells [7].

97 There is epidemiologic evidence that children with sickle cell trait are protected from severe
98 manifestations of malaria in malaria endemic areas [8-10]. HbAS has been shown to protect
99 against severe malarial anaemia, high density parasitemia and to be associated with reduced all
100 cause mortality between the ages of 2 to 16 months [11]. This also happens to be the age when
101 children have the highest episodes of severe malarial anaemia [11]. The balanced
102 polymorphism in the HbS-malaria relationship is thought to be maintained by higher mortality
103 risk of HbAAs due to malaria and high mortality risk of HbSSs caused by complications of HbSS
104 [12]. The HbS allele apparently does not prevent infection but results in impaired entry and
105 growth of the parasites during the erythrocytic stage of development [13-16], enhanced removal
106 of the parasitized variant RBCs [17] and reduced rosette formation [18]. When heterozygotes
107 are infected, the merozoites, which have a high metabolic rate, consume a lot of oxygen and
108 this leads to sickling under low oxygen tension. The spleen removes these sickled cells before
109 the micro-organisms have a chance to produce a large infectious population in the body. It is

this selective heterozygote advantage that maintains HbS gene at a higher level in malarial than in non-malarial environments [12, 17]. The prevalence of the sickle cell trait in Western Kenya is estimated to be approximately 17.4% [11]. This high level of sickle cell carrier state in this region is thought to be maintained by the high malaria prevalence [12].

1.1. Malaria, Sickle Cell Trait and the Complement System

Erythrocyte complement regulatory proteins, CR1, CD55, and CD59 may protect RBCs from complement mediated damage that occur when immune complexes are formed during malaria infection [3, 19]. Children with severe malaria associated anaemia have been shown to have reduced immune complex binding capacity and increased susceptibility of their red cells to immune complex deposition and this may predispose their erythrocytes to complement mediated damage and phagocytosis [20]. On the other hand, HbAS individuals have been shown to have fewer episodes of severe malaria associated anaemia, reduced risk of anaemia episodes and reduced risk of high density parasitemia than their counterparts with HbAA [11].

Severe *P. falciparum* malaria leads to the formation of immune complexes in the peripheral circulation and this increased circulation of immune complexes has been associated with the development of severe anaemia [2, 21]. CR1 and CD55 protect RBCs from complement mediated damage that can occur when immune complexes are formed during malaria infection [19].

Given the important role of RBCs in removal of immune complexes from circulation and in regulation of complement activation, it is therefore important to consider the competence of sickle cell trait RBCs in these functions. So far, the level of CR1 and CD55, the immune complex binding capacity and the susceptibility to complement in heterozygous carriers of the sickle cell gene has not been studied. There is strong evidence that the complement regulatory system machinery is important in the protection from the severe manifestations of malaria. The aim of this study was therefore, to study the same machinery in sickle cell trait to determine whether this could explain the protection from severe malaria.

Complement activation plays an important role in immune defense against malaria infection [22-24] and may also contribute to the disease severity if excessively activated [22, 25, 26] or if inappropriately controlled [27]. Indeed, complement factors have been associated with cerebral

malaria [23, 25] and implicated in the pathogenesis of severe malarial anemia (SMA) through mechanisms leading to increased destruction of uninfected as well as infected RBCs [27-30].

Erythrocytes are normally protected from autologous complement attack through the action of various complement regulatory proteins, such as complement receptor 1 (CR1/CD35), decay accelerating factor (DAF/CD55) and membrane inhibitor of reactive lysis (CD59) expressed on these cells [31]. Recent studies have demonstrated age-related changes in the expression levels of the erythrocyte complement regulatory proteins; CR1 and CD55 [32] and associated deficiencies of these molecules with SMA [2]. Although complement regulatory proteins play an important role in preventing complement mediated erythrocyte destruction, they have not been investigated in association with protection of sickle cell traits against SMA. In this study, it was hypothesized that erythrocytes of individuals with sickle cell trait may have increased CR1 per erythrocyte and CD55 (DAF) which in turn causes increased rate of immune complexes clearance from the circulation and hence resistance to complement attack. To test this hypothesis, RBCs of individuals with HbAS and those with HbAA were examined for differences in CR1 and CD55 expression levels under both normal and reduced oxygen saturation from a cohort of individuals living in a malaria endemic region of western Kenya.

2. MATERIALS AND METHODS

2.1. Study site and design

The study site was Kombewa Division, in Kisumu West District, Nyanza Province, in Western Kenya. Kombewa is situated about 35 kilometres west of Kisumu town and has previously been used as a site for many epidemiological studies in both adults and children [33, 34].

Kombewa borders Lake Victoria and has a population of about 65,000 people. Malaria transmission in this area occurs all year round with peak seasons following the long rains (March to May) and the short rains (October to December). The annual inoculation rates are estimated to be 31.1 infective bites per person per year [34].

2.2 Study population

Forty seven (47) HbAS individuals aged 0-192 months were matched to 69 individuals with HbAA of similar age (± 2 months or ± 24 months for those below or more than 96 months, respectively) at a ratio of 1:1 or 1:2. Since many acute or chronic conditions including malaria [2, 3, 27, 35], HIV infection [36] and others [37] are known to interfere with the parameters under investigation such as complement regulatory proteins, the exclusion criteria included evidence

of malnutrition, immuno compromised status, severe anemia (Haemoglobin $\leq$ 5.0 g/dL); bacterial infection such as pneumonia; malignancy; and blood transfusion within 3 months preceding the study. In cases of an acute illness, the potential participants were assessed, treated and asked to come again for re-evaluation. At re-evaluation, the potential participants were enrolled when they were deemed well.

2.3 Ethical consideration

Recruitment of study participants and procedures were in accordance with all applicable regulations. Informed consent was obtained from all participants or parents/guardians of children. This study was reviewed and approved by the Kenya National Ethical Review Committee of the Kenya Medical Research Institute and by the Human Subjects Research Review Board of the Office of the Surgeon General, U.S. Army.

2.4 Deoxygenation of the RBCs for Assay

An equal amount of RBCs in wash buffer was added to freshly prepared disodium hydrogen phosphate (Na_2HPO_4 , FW 142g) 0.114M and sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$ FW 174.1g) 0.114M at a ratio of 2:3, filter sterilized through a 0.22 μm filter [18]. The disodium hydrogen phosphate was prepared from a stock solution while the sodium dithionite was prepared fresh every day. The RBCs were incubated at 37°C for 1 hour and then washed twice with wash buffer before running the assays side by side. This duration of treatment with the dithionite was found to give the maximal sickling for HbAS RBCs. This procedure was done to see the effect of reduced oxygen saturation on the parameters under investigation

2.5 Staining of Red Blood Cells for CR1 and CD55

Venous blood (2.5 mL) was collected in ethylenediaminetetraacetic acid (EDTA) vacutainers (Becton Dickinson, USA). A 1:50 dilution of RBC pellet in Alsevers buffer was made from EDTA anticoagulated blood samples, centrifuged at x500g for five minutes and a portion resuspended in the same volume of buffer for storage at 4° C until used. The remaining pellet was washed with ten volumes of phosphate buffered saline pH 7.4 (PBS) and resuspended in the glycerolyte (50% glycerol, 16g/L sodium lactate, 300mg/L KCL, 25mM sodium phosphate pH 6.8) for cryo-preservation [38], stored at -70° C freezer overnight then transferred to liquid nitrogen until use.

Since our preliminary experiments reported no significant effect of freezing on the levels of erythrocyte CR1 and CD55, current investigations were carried out using cryo-preserved samples. For the determination of CR1 and CD55, the following primary antibodies were used in dilution of 1:20: anti-CR1 clone E11, anti-CD55 clone IA10, and isotype controls for each (Becton-Dickinson, Belgium). A secondary FITC-conjugated goat anti-mouse IgG (Becton-Dickinson, Belgium) was used at a dilution of 1:50. Ten μ L of thawed erythrocyte pellet was washed twice in 1mL of Alsevers buffer and resuspended in the same volume of buffer. The rest of the procedures were done as previously described [3, 32]

2.6 Flow Cytometric Analysis

Flow cytometry was carried out using a FACScan cytometer (Becton-Dickinson, San Jose, CA, USA). Analysis was carried out using FCS Express v2.5 (De Novo Software, Los Angeles, CA). Red cells were gated on the basis of their forward and side scatter characteristics using logarithmic amplification. As an internal measure of consistency, all assays were done in parallel to RBCs from a healthy aparasitemic subject who acted as a positive control for complement regulatory proteins. The mean fluorescence intensity (MFI) values for CR1 and CD55 were normalized to the mean of the MFI of the red cell standard using the formula

$$\text{CorrMFIs} = \text{MFIs} \times \text{MFIcmean}/\text{MFIc},$$

Where “CorrMFIs and MFIs are the corrected and uncorrected sample MFI respectively, ‘MFIcmean” is the mean of all the MFI values of the standard control, and “MFIc” is the MFI of the control obtained in parallel with the sample.

The number of molecules of CR1 per red cell was derived from a fluorescence standard curve created using cells with known CR1 numbers. Red cell anti-CD55 antibody binding capacities (ABC) were derived from a standard curve created using beads of known ABC (Bangs Lab, Fishers, IN) [39]

2.7 Statistical Analysis

Statistical analyses were performed using SPSS for windows version 16.0 software (SPSS Inc, Chicago, IL, USA). The mean CR1 copy numbers per red cell and mean CD55 levels per red cell data are presented graphically for each age group as box plots, where the box represents boundaries between the 25th and 75th percentile, the line through the box represents median

and whiskers the 10th and 90th percentile limits. Analysis of variance (ANOVA) was used to detect differences across age groups adjusting for factors and covariates. The independent samples *t*-test was used for comparisons of normal continuous data between two groups, while the Chi-square (χ^2) and Mann-Whitney U tests were utilized to examine differences between proportions and for pair wise comparisons of medians, respectively. Bivariate logistic regression analysis was carried out to determine the Odds Ratio (OR) and the 95% confidence interval (CI) for CR1 copy numbers per erythrocyte and mean CD55 levels per red cell between HbAS and HbAA. The General Linear Method (GLM) was used to test between subject effects. The Chi-square (χ^2) test was used to compare proportions across groups. All tests were two-sided with $\alpha \leq 0.05$.

241

242 **3. RESULTS**

243

244 **3.1 Demographics**

245

246 Forty seven (47) and 69 children aged 0 to 192 months were enrolled in the HbAS and HbAA
247 arms respectively as shown in table 1. There was no significant difference in gender, mean age
248 in months and mean haemoglobin level between the two groups ($P= 0.447$, $P= 0.059$ and $P=$
249 0.548 respectively). The mean CR1 level and mean antibody binding capacity under both
250 normal and reduced oxygen saturation were comparable ($P = 0.2432$, 0.3651 , 0.8155 and
251 0.1700 respectively)

252

253

254

255

256

Table 1 Demographic, Clinical and Laboratory Characteristics of the Study Population

Characteristic	HbAA (n = 69)	HbAS (n = 47)	P
Gender			
Male n (%)	28 (54.9)	23 (45.1)	0.447 ^a
Female n (%)	41 (63.1)	24 (36.9)	
Mean Age in months (95% CI)	56.6 (37.3-75.9)	98.1 (53.5-142.8)	0.059 ^a
Haemoglobin levels in g/dL (95% CI)	10.7 (10.3-11.2)	10.9 (10.5-11.4)	0.548 ^a
Mean CR1 copy #/Erythrocyte (95% CI)	471.6 (431.9-511.4)	509.4 (457.4-561.4)	0.346 ^b
Mean CR1 copy #/Erythrocyte Deoxygenated (95% CI)	605.3 (561.0-649.6)	575.9 (532.1-619.7)	0.321 ^b
Mean CD55 ABC/Erythrocyte (95% CI)	2520.02 (2331.36-2708.68)	2671.4 (2388.0-2954.8)	0.813 ^b
Mean CD55 ABC/Erythrocyte Deoxygenated (95% CI)	2590.8 (2373.2-2808.4)	2934.2 (2609.1-3259.2)	0.169 ^b
Numeric P. falciparum read			
Negative n (%)	38 (61.3)	24 (38.7)	0.672
Positive n (%)	31 (57.4)	23 (42.6)	

257

258

259

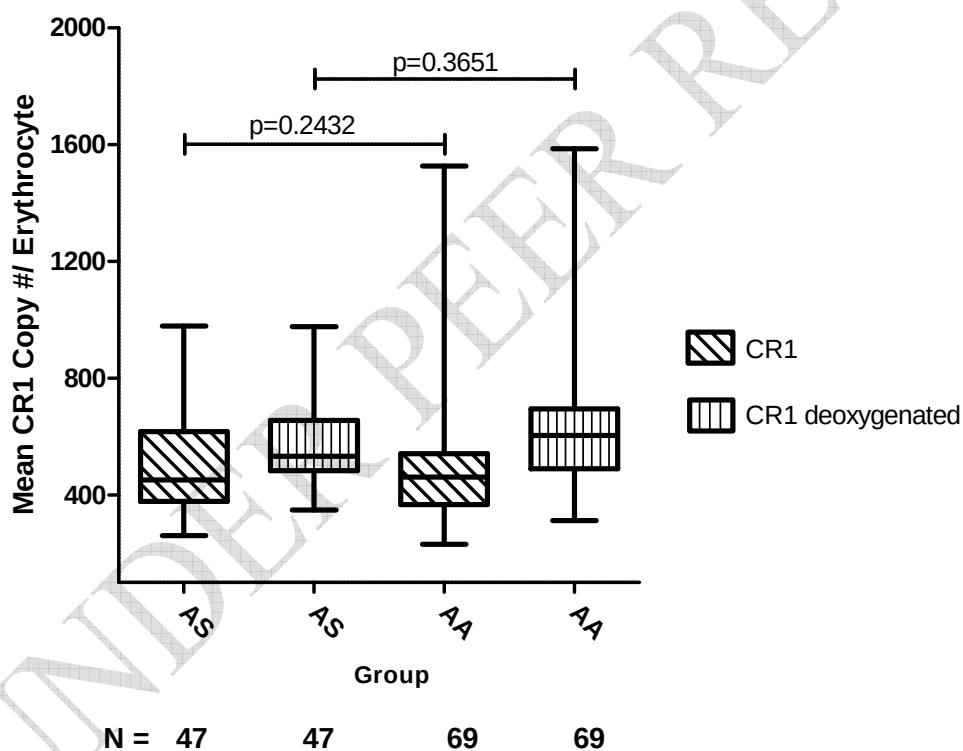
260

Data are presented as means (95% CI). Children (n=116) were categorized according to the haemoglobin types into either HbAA (n=69) or HbAS (n=47). a:Statistical significance determined by the Chi-square analysis. b Statistical significance determined by Mann-Whitney U test.

261

262 3.2 Differences between Mean CR1 Copy Numbers between HbAS and HbAA Cells 263 under normal and reduced Oxygen Saturation

264 The mean CR1 copy numbers before and after treatment with sodium dithionite are presented
265 as box-and-whisker plots. The mean CR1 copy numbers per red blood cell was higher (509,
266 [SD=177]) in the HbAS group than in the HbAA group (471 [SD=165]), this difference was
267 statistically not significant ($P = 0.243$). When RBCs were treated with sodium dithionite, the
268 mean CR1 copy numbers per erythrocyte were higher in both groups, for HbAS (576, [SD=149]
269 and for HbAA (605, [SD=184] but again, there was no statistically significant difference between
270 the two groups ($P = 0.365$) (Figure 1)



271

272 Figure 1 CR1 copy numbers between HbAS and HbAA red cells under normal and
273 reduced Oxygen Saturation

274

275

276

277

3.3 Differences in CR1 Copy Numbers between HbAS and HbAA Red Cells in each Age Cohort

The CR1 copy numbers per erythrocyte (Figure 2) was observed to be significantly higher in the 49-192 months age group, HbAS [mean = 596.6, SD 168] and HbAA [mean = 468.6, SD = 99], $P = 0.033$. Overall, significant positive correlation was observed between the CR1 copy numbers per erythrocyte with age for all individuals in the HbAS and HbAA groups, $r(116) = 0.190$, $P = 0.041$, CD55 levels: $r(116)=0.417$, $P=0.000$ and CD 55 levels after deoxygenation: $r(116).=0.451$, $P=0.000$.

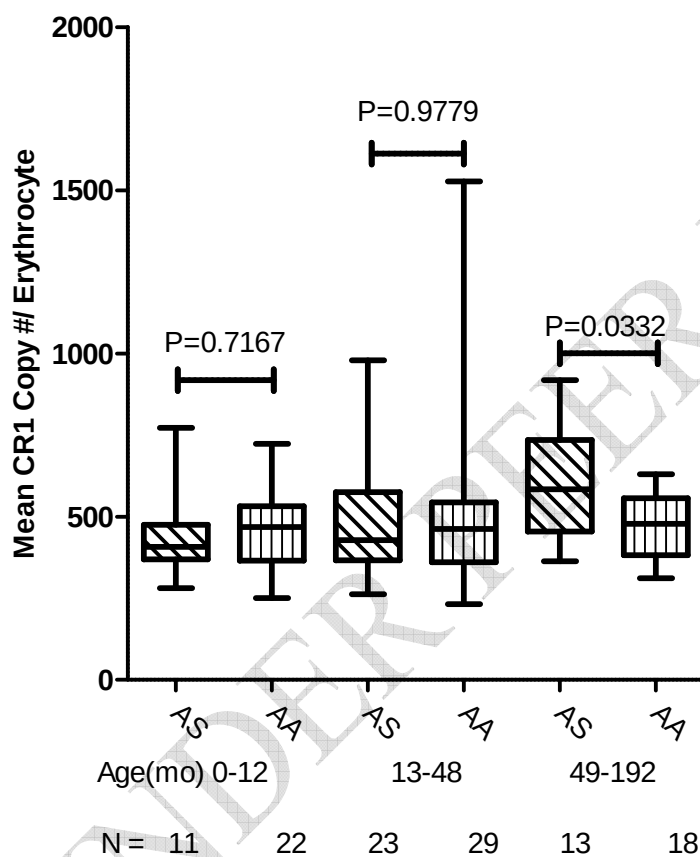


Figure 2: Mean CR1 Copy Numbers for HbAS and HbAA Red Cells across the various Age Cohorts

3.4 Mean CR1 Copy Numbers per Erythrocyte for the various Age Cohorts by Haemoglobin Electrophoresis Results under reduced Oxygen Saturation

The CR1 copy numbers per erythrocyte was observed to vary with age for both HbAS and HbAA groups (figure 3). The mean CR1 copy numbers per erythrocyte after treatment with sodium dithionite for the various age cohorts are presented. Under reduced oxygen saturation, the mean CR1 copy numbers per erythrocyte was observed to be higher in the HbAA compared to the HbAS but this was not statistically significant. After the age of 96 months, the mean CR1 copy numbers per erythrocyte was higher in the HbAS compared to the HbAA but again, this was not statistically significant.

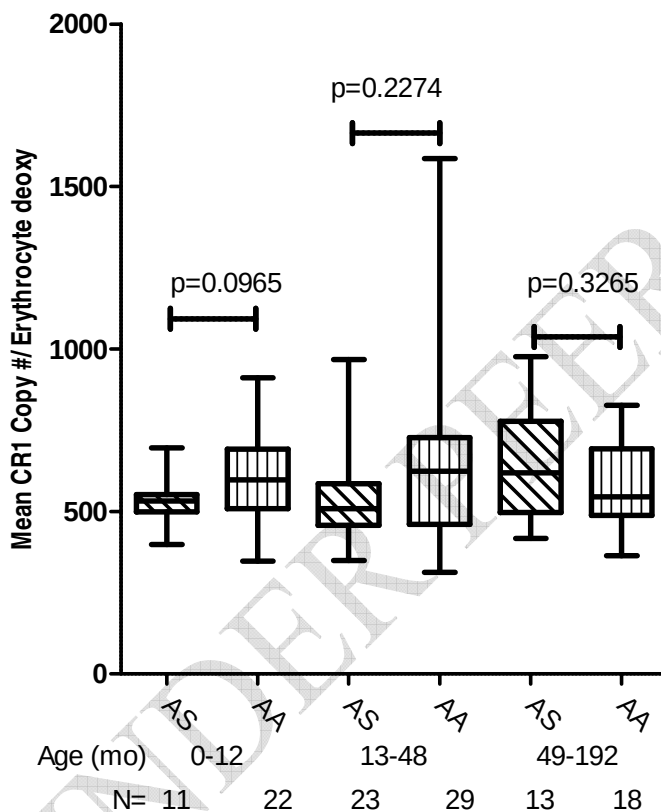


Figure 3: Mean CR1 Copy Numbers for HbAS and HbAA Red Cells across the various Age Cohorts under reduced oxygen saturation

3.5 Differences in Mean CD levels between HbAS and HbAA Red Cells under normal and reduced Oxygen Saturation

There were no significant differences in the mean CD55 ABC between red cells of individuals with HbAS and those with HbAA. Mean CD55 ABC of HbAS cells (2671, [SD 149]) was higher than HbAA cells (2541, [SD 777]), However, this difference was not statistically significant $P = 0.816$. Under reduced oxygen saturation, the mean CD55 ABC for the HbAS (2934, [SD 1107]) was higher than for HbAA group (2591, [SD 906]). This again did not reach statistical significance ($P = 0.1700$) (Figure 5).

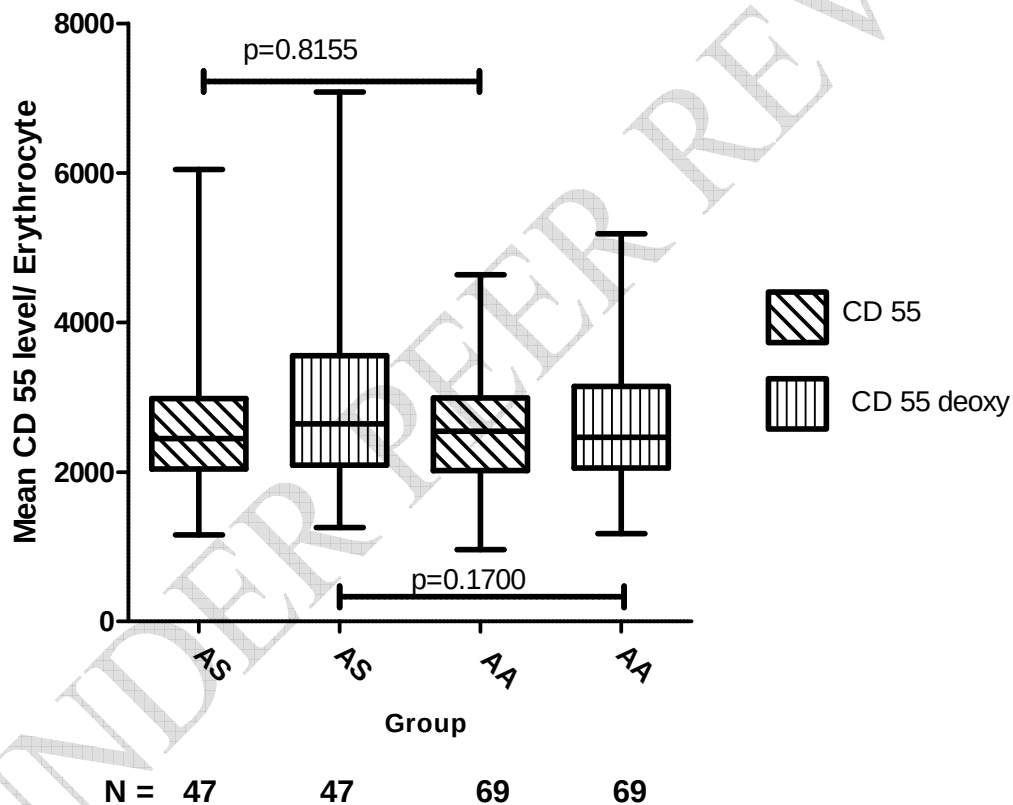


Figure 4: Mean CD levels of HbAS and HbAA Red Cells under normal and reduced Oxygen Saturation

3.5.1 Differences in Mean CD levels of HbAS and HbAA Red Cells in each Age Cohort

There was no statistically significant difference between the mean CD55 level per erythrocytes between HbAS and HbAA in the 0-12, 13-48 and 49-192 months age cohorts ($P= 0.6028$, 0.7746 and 0.2539 respectively) (Figure 5)

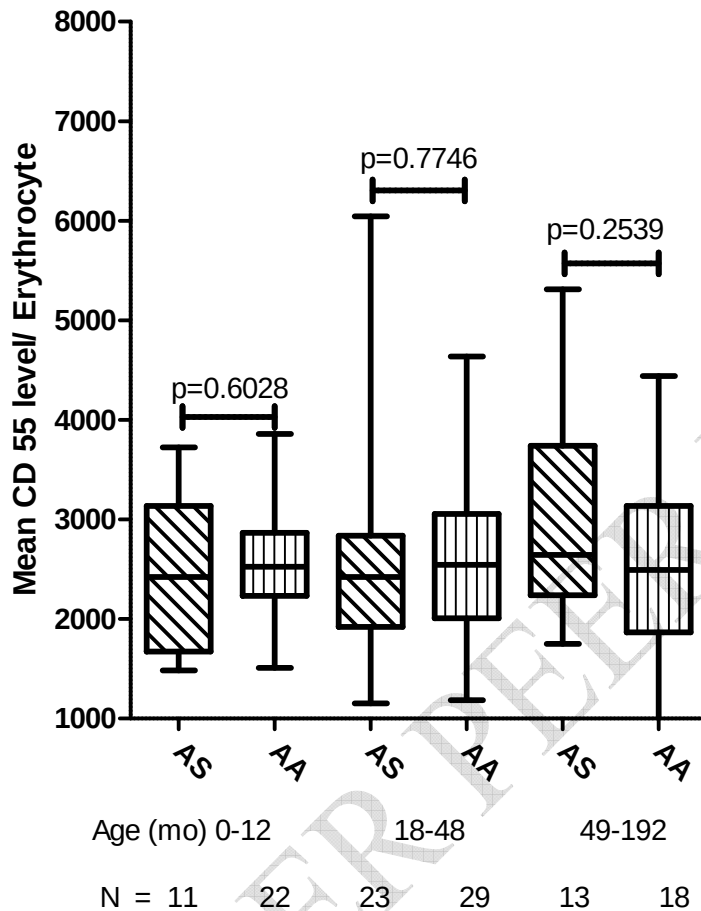


Figure 5: Mean CD levels for the various Age Cohorts

3.5.2 Mean CD levels for the various Age Cohorts under Reduced Oxygen saturation

Reduced oxygen saturation resulted in greater increase in the mean CD55 ABC for the HbAS erythrocytes between the ages of 49-192 months. The mean CD55 ABC for the HbAS was 2934 (SD 1107) compared to HbAA which was 2591 (SD 906). This difference was statistically significant ($P=0.011$)

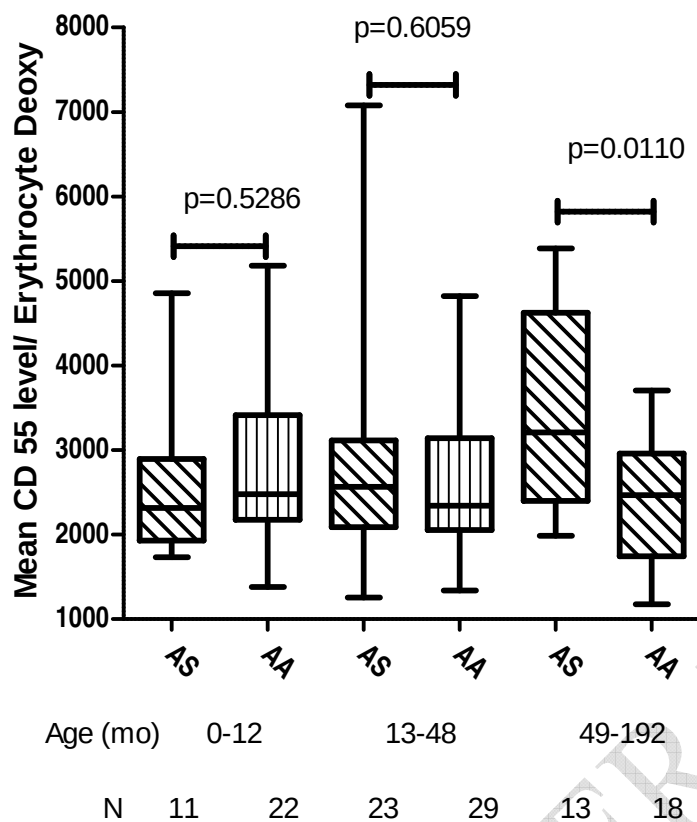


Figure 6: Mean CD levels for the various Age Cohorts under reduced Oxygen Saturation

4. DISCUSSION

This study has shown that the mean CR1 copy numbers per erythrocyte was significantly higher in the HbAS than in the HbAA individuals only in the 49-192 months age cohort ($P = 0.0332$) under normal oxygenation. It is possible that beyond the age of 49 months, the high mean CR1 copy numbers per erythrocyte could translate to higher immune complex binding capacity and decreased immune complex deposition on the red blood cells and this could protect the red cells from immune complex mediated destruction. Although the mean CD55 ABC for the HbAS and HbAA were comparable in the various age cohorts under normal oxygenation, the mean CD55 ABC for the HbAS in the 49-192 months age cohort after deoxygenation was much higher in the HbAS (mean 3466[SD1092]) than in the HbAA (mean 2373[SD752]). This difference was

statistically significant ($P=0.011$). We postulate that the apparent increase in CD55 under low oxygenation may be due to conformational changes in CD55 and this may result in decreased complement susceptibility of these cells.

Natural resistance to malaria based on the inheritance of selected genes that confer protection has been observed in a few individuals living in malaria endemic areas [40, 41]. The relative protection of the sickle cell trait against clinical malaria increases throughout the first 10 years of life, returning thereafter to baseline [42]. Transgenic mice expressing sickle cell haemoglobin (HbS) are protected from rodent malaria [16] and rodent cerebral malaria [43]. In humans HbS provides selective protection to heterozygous sickle cell trait individuals (HbAS) against death from *Plasmodium falciparum* malaria while homozygous sickle cell trait individuals (HbSS) suffer severe clinical effects [12, 44-46]. Studies carried out in The Gambia [14]) and Kenya [47, 48] have indicated that HbAS provides more than 90% protection from both cerebral malaria and severe malarial anemia in children. The mechanism of malaria protection by HbAS is not well understood but has been suggested to be due to enhancement of acquired immunity to the parasite [42, 49] and innate factors such as impaired parasite growth in erythrocytes containing HbAS attributable to conditions of low oxygen tension [50]. A recent study [51] in the same population has shown that the protection afforded by HbAS against severe manifestations of malaria may be partly due to higher immune complex binding capacity of the HbAS compared to the HbAA red cells and this could result in protection of these cells against malaria. This high immune complex binding capacity could be partly due to the high CR1 levels and the high CD55 levels when these cells undergo deoxygenation

5. CONCLUSIONS

In conclusion, we report for the first time that the mean CR1 copy numbers per erythrocyte is higher in the HbAS than HbAA individuals between the ages of 49-192 months. When the cells were subjected to deoxygenation, the level of CD55 was greater in HbAS RBCs than in HbAA RBCs between 49-192 months of age. This is most likely due to conformational changes of the CD55 molecule leading to increased availability of the epitope. this apparently has an effect on the immune complex binding capacity of these cells and may partially explain the protection afforded by the HbAS cells against severe manifestations of malaria.

These results taken together may translate into protection of the HbAS individuals against severe manifestations of malaria.

Competing interests

There is no conflict of interest for any of the authors of the manuscript due to commercial or other affiliations. The study was approved by the ethical and scientific review committees at the Kenya Medical Research Institute and the institutional review board at the Walter Reed Army Institute of Research

Authors' contributions

WO helped in study design, conducted all experiments, helped in data analysis and drafted the manuscript, JAS designed the study, directed the work, drafted the manuscript, helped in data analysis and data interpretation, BBAE helped in study design, data analysis, data interpretation and helped in drafting of the manuscript, JRA helped in study design, data analysis, data interpretation and helped in drafting of the manuscript, MMO helped in study design, data analysis, data interpretation and helped in drafting of the manuscript, SMOG helped in study design, data analysis, data interpretation and helped in drafting of the manuscript.

Disclaimer

The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official, or as reflecting true views of the Department of Defense

Acknowledgments

We are indebted to the Walter Reed Project/KEMRI staff for clinical support and to the parents/guardians of the study participants and the children that participated in the study. These data are published with the approval of the Director, Kenya Medical Research Institute (KEMRI). Financial support: work was partly supported by a training grant from the Fogarty International Center (1 D43 TW06239, PI José A. Stoute) and NIH HL75102 (PI José A. Stoute).

REFERENCES

1. Rowe JA, Moulds JM, Newbold CI, Miller LH. P. falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature*. 1997;388:292-5.
2. Stoute JA, Odindo AO, Owuor BO, Mibei EK, Opollo MO, Waitumbi JN. Loss of red blood cell-complement regulatory proteins and increased levels of circulating immune complexes are associated with severe malarial anemia. *J Infect Dis*. 2003;187(3):522-5.
3. Waitumbi JN, Opollo MO, Muga RO, Misore AO, Stoute JA. Red cell surface changes and erythrophagocytosis in children with severe plasmodium falciparum anemia. *Blood*. 2000;95(4):1481-6.
4. Cserti CM, Dzik WH. The ABO blood group system and Plasmodium falciparum malaria. *Blood*. 2007;110(7):2250-8.
5. Rowe A, Obeiro J, Newbold CI, Marsh K. Plasmodium falciparum rosetting is associated with malaria severity in Kenya. *Infect Immun*. 1995;63(6):2323-6.
6. Cockburn IA, Mackinnon MJ, O'Donnell A, Allen SJ, Moulds JM, Baisor M, et al. A human complement receptor 1 polymorphism that reduces Plasmodium falciparum rosetting confers protection against severe malaria. *Proc Natl Acad Sci U S A*. 2004;101(1):272-7.
7. Nagayasu E, Ito M, Akaki M, Nakano Y, Kimura M, Looareesuwan S, et al. CR1 density polymorphism on erythrocytes of falciparum malaria patients in Thailand. *Am J Trop Med Hyg*. 2001;64(1-2):1-5.
8. Dvorak JA, Miller LH, Whitehouse WC, Shiroishi T. Invasion of erythrocytes by malaria merozoites. *Science*. 1975;187(4178):748-50.
9. Ganczakowski M, Bowden DK, Maitland K, Williams TN, O'Shaughnessy D, Viji J, et al. Thalassaemia in Vanuatu, south-west Pacific: frequency and haematological phenotypes of young children. *Br J Haematol*. 1995;89(3):485-95.
10. Ruwende C, Khoo SC, Snow RW, Yates SN, Kwiatkowski D, Gupta S, et al. Natural selection of hemi- and heterozygotes for G6PD deficiency in Africa by resistance to severe malaria. *Nature*. 1995;376(6537):246-9.
11. Aidoo. Protective effects of the sickle cell gene against malaria morbidity and mortality. *Lancet*. 2002;359:1311-12.
12. Aluoch JR. Higher resistance to Plasmodium falciparum infection in patients with homozygous sickle cell disease in western Kenya. *Trop Med Int Health*. 1997;2(6):568-71.
13. Chippaux JP, Massougbodji A, Boulard JC, Akogbeto M. [Morbidity and severity of malaria attacks in carriers of sickle-cell trait]. *Rev Epidemiol Sante Publique*. 1992;40(4):240-5.
14. Hill AV, Allsopp CE, Kwiatkowski D, Anstey NM, Twumasi P, Rowe PA, et al. Common west African HLA antigens are associated with protection from severe malaria. *Nature*. 1991;352(6336):595-600.
15. Pasvol G, Weatherall DJ, Wilson RJ. Cellular mechanism for the protective effect of haemoglobin S against P. falciparum malaria. *Nature*. 1978;274(5672):701-3.
16. Shear HL, Roth EF, Jr., Fabry ME, Costantini FD, Pachnis A, Hood A, et al. Transgenic mice expressing human sickle hemoglobin are partially resistant to rodent malaria. *Blood*. 1993;81(1):222-6.
17. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. *Bull World Health Organ*. 2001;79(8):704-12.
18. Carlson J, Nash GB, Gabutti V, al-Yaman F, Wahlgren M. Natural protection against severe Plasmodium falciparum malaria due to impaired rosette formation. *Blood*. 1994;84(11):3909-14.

- 459 19. Jhaveri KN, Ghosh K, Mohanty D, Parmar BD, Surati RR, Camoens HM, et al.
460 Autoantibodies, immunoglobulins, complement and circulating immune complexes in acute
461 malaria. *Natl Med J India*. 1997;10(1):5-7.
- 462 20. Owuor BO, Odhiambo CO, Otieno WO, Adhiambo C, Makawiti DW, Stoute JA.
463 Reduced immune complex binding capacity and increased complement susceptibility of red cells
464 from children with severe malaria-associated anemia. *Mol Med*. 2008;14(3-4):89-97.
- 465 21. Mibei EK, Orago AS, Stoute JA. Immune complex levels in children with severe
466 *Plasmodium falciparum* malaria. *Am J Trop Med Hyg*. 2005;72(5):593-9.
- 467 22. Nyakoe NK, Taylor RP, Makumi JN, Waitumbi JN. Complement consumption in
468 children with *Plasmodium falciparum* malaria. *Malar J*. 2009;8(1):7.
- 469 23. Odera M, Otieno W, Adhiambo C, Stoute JA. Dual role of erythrocyte complement
470 receptor type 1 in immune complex-mediated macrophage stimulation: implications for the
471 pathogenesis of *Plasmodium falciparum* malaria. *Clinical and experimental immunology*.
472 2011;166(2):201-7.
- 473 24. Wenisch C, Spitzauer S, Florris-Linau K, Rumpold H, Vannaphan S, Parschalk B, et al.
474 Complement activation in severe *Plasmodium falciparum* malaria. *Clin Immunol Immunopathol*.
475 1997;85(2):166-71.
- 476 25. Adam C, Geniteau M, Gougerot-Pocidalo M, Verroust P, Lebras J, Gibert C, et al.
477 Cryoglobulins, circulating immune complexes, and complement activation in cerebral malaria.
478 *Infect Immun*. 1981;31(2):530-5.
- 479 26. Thomas BN, Diallo DA, Noumsi GT, Moulds JM. Circulating Immune Complex Levels
480 are Associated with Disease Severity and Seasonality in Children with Malaria from Mali.
481 *Biomark Insights*. 2012;7:81-6.
- 482 27. Stoute JA. Complement-regulatory proteins in severe malaria: too little or too much of a
483 good thing? *Trends Parasitol*. 2005;21(5):218-23.
- 484 28. Kai OK, Roberts DJ. The pathophysiology of malarial anaemia: where have all the red
485 cells gone? *BMC Med*. 2008;6:24.
- 486 29. Rout R, Dhangadamajhi G, Mohapatra BN, Kar SK, Ranjit M. High CR1 level and
487 related polymorphic variants are associated with cerebral malaria in eastern-India. *Infect Genet*
488 *Evol*. 2011;11(1):139-44.
- 489 30. Stoute JA. Complement receptor 1 and malaria. *Cell Microbiol*. 2011;13(10):1441-50.
- 490 31. Abul KA, Andrew HL, Jordan SP. Saunders Texts and Review Series;Cell and Molecular
491 Immunology. 1994;Fourth Edition.
- 492 32. Waitumbi JN, Donvito B, Kisserli A, Cohen JH, Stoute JA. Age-related changes in red
493 blood cell complement regulatory proteins and susceptibility to severe malaria. *J Infect Dis*.
494 2004;190(6):1183-91.
- 495 33. Kifude CM, Polhemus ME, Heppner DG, Jr., Withers MR, Ogutu BR, Waitumbi JN. Hb
496 Kenya among Luo adults and young children in malaria holoendemic Western Kenya: screened
497 by high performance liquid chromatography and confirmed by polymerase chain reaction.
498 *Hemoglobin*. 2007;31(4):401-8.
- 499 34. Ndenga B, Githeko A, Omukunda E, Munyekenye G, Atieli H, Wamai P, et al.
500 Population dynamics of malaria vectors in western Kenya highlands. *J Med Entomol*.
501 2006;43(2):200-6.
- 502 35. Roestenberg M, McCall M, Mollnes TE, van Deuren M, Sprong T, Klasen I, et al.
503 Complement activation in experimental human malaria infection. *Trans R Soc Trop Med Hyg*.
504 2007;101(7):643-9.

36. Aries SP, Schaaf B, Hansen F, Weyrich K, Kurowski V, Dennin R, et al. Expression of complement receptors and regulatory proteins on alveolar CD4⁺ lymphocytes from human immunodeficiency virus-1 infected individuals. *Eur Respir J*. 1997;10(8):1736-41.
37. Spendlove I, Ramage JM, Bradley R, Harris C, Durrant LG. Complement decay accelerating factor (DAF)/CD55 in cancer. *Cancer Immunol Immunother*. 2006;55(8):987-95.
38. Cockburn IA, Donvito B, Cohen JH, Rowe JA. A simple method for accurate quantification of complement receptor 1 on erythrocytes preserved by fixing or freezing. *J Immunol Methods*. 2002;271(1-2):59-64.
39. Schwartz A, Ottinger J, Wallace E, Poon R, Fernandez-Repollet E. Quantitative determination of antibody binding capacity (ABC) by flow cytometry. *Eur J Histochem*. 1994;38 Suppl 1:13-20.
40. Weatherall DJ. Common genetic disorders of the red cell and the 'malaria hypothesis'. *Ann Trop Med Parasitol*. 1987;81(5):539-48.
41. Williams TN. Human red blood cell polymorphisms and malaria. *Curr Opin Microbiol*. 2006;9(4):388-94.
42. Williams TN, Mwangi TW, Roberts DJ, Alexander ND, Weatherall DJ, Wambua S, et al. An immune basis for malaria protection by the sickle cell trait. *PLoS Med*. 2005;2(5):e128.
43. Kaul DK, Nagel RL, Llena JF, Shear HL. Cerebral malaria in mice: demonstration of cytoadherence of infected red blood cells and microrheologic correlates. *Am J Trop Med Hyg*. 1994;50(4):512-21.
44. Aluoch JR. The presence of Sickle Cells in the peripheral blood film. Specificity and sensitivity of diagnosis of homozygous Sickle Cell disease in Kenya. *Trop Geogr Med*. 1995;47(2):89-91.
45. Nagel RL, Fleming AF. Genetic epidemiology of the beta s gene. *Baillieres Clin Haematol*. 1992;5(2):331-65.
46. Otieno W EB, Aluoch JR, Gondi SMO and Stoute JA. Association between Sickle Cell Trait and Low Density Parasitaemia in a *P. falciparum* Malaria Holoendemic Region of Western Kenya. *International Journal of TROPICAL DISEASE & Health*. 2012;2(4):231-40.
47. Marsh K. Malaria--a neglected disease? *Parasitology*. 1992;104 Suppl:S53-69.
48. Williams TN, Mwangi TW, Wambua S, Alexander ND, Kortok M, Snow RW, et al. Sickle cell trait and the risk of *Plasmodium falciparum* malaria and other childhood diseases. *J Infect Dis*. 2005;192(1):178-86.
49. Verra F, Simpori J, Warimwe GM, Tetteh KK, Howard T, Osier FH, et al. Haemoglobin C and S role in acquired immunity against *Plasmodium falciparum* malaria. *PLoS ONE*. 2007;2(10):e978.
50. Pasvol G. The interaction between sickle haemoglobin and the malarial parasite *Plasmodium falciparum*. *Trans R Soc Trop Med Hyg*. 1980;74(6):701-5.
51. Otieno W EB, Odera MM, Aluoch JR and Stoute JA. Red Blood Cell Immune Complex Binding Capacity in Children with Sickle Cell Trait (HbAS) Living in *P. falciparum* Malaria Holoendemic Region of Western Kenya. *International Journal of TROPICAL DISEASE & Health* 2012;2(4):272-82.